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Antibacterial Properties of Isolated Amoebocytes From the Sea Anemone *Actinia equina*

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Abstract. The antimicrobial defenses of anthozoans were investigated *in vitro* by extracting amoebocytes from the mesenteric filaments of the beadlet anemone, *Actinia equina*, and testing for their ability to phagocytose and kill the gram-negative bacterium *Psychrobacter immobilis*. Only the hyaline amoebocytes exhibited phagocytosis *in vitro*, with about 40% seen to ingest one or more bacteria over 45 min. Mixed cultures of viable amoebocytes were further found to produce O_2^- ions and other reactive oxygen species (ROS) after stimulation with phorbol myristate acetate or lipopolysaccharide. Co-incubation of viable amoebocytes with *P. immobilis* for 3 h *in vitro* resulted in reduced growth of the bacterium compared to saline-incubated bacteria, but because the growth of *P. immobilis* was also impaired by lysed control amoebocytes, the contribution made to bacterial killing by ROS could not be evaluated. Instead, as confirmed by additional experiments using lysate supernatants of the amoebocytes, it appears that the cells contain soluble bactericidal factors. The nature of these agents is at present unknown, although preliminary tests indicate that killing is not mediated by lysozyme.

Introduction

The Cnidaria is a large, diverse, and ecologically important phylum. It includes about 9400 species, of which 68% are members of the class Anthozoa (Banister and Campbell, 1985). In common with all animals, anthozoans need to protect themselves against the lethal or debilitating consequences of microbial or parasitic invasion. Indeed, a recent report offers evidence that bleaching, one of the most destructive pathological conditions affecting reef corals, may be caused by bacterial infection (Kushmaro *et al.*, 1996). Concern about the health of ecologically important anthozoans, mainly scleractinian corals (Grigg and Dollar, 1990), has stimulated interest in the way these animals overcome or avoid opportunistic or pathogenic infection. However, unlike their coelomate relatives, anthozoans have received little attention with respect to their anti-microbial and anti-parasitic defenses.

The ability of anthozoans to display discriminatory tissue reactions to foreign grafts has been demonstrated by many workers, including Theodor (1970), Burnet (1971), Bigger and Hildemann (1982), Porter and Targett (1988), Jokiel and Bigger (1994), and Rinkevitch *et al.* (1994). It is also widely accepted that these animals possess populations of wandering amoebocytes within the mesoglea (Young, 1974; Patterson and Landolt, 1979; Van der Vyver, 1981; Shick, 1991), and a few studies have noted that these cells tend to migrate to wounds or grafts *in vivo* (Young, 1974; Patterson and Landolt, 1979; Bigger and Olano, 1994). However, despite the rarity of bacterial infection or parasitization in the tissues of these animals (Patterson and Landolt, 1979; Bigger and Hildemann, 1982), detailed *in vitro* analyses of the phagocytic capability of the amoebocytes in anthozoans have not been undertaken, and the cellular basis of antibacterial activity remains largely unknown.

The present study was aimed at determining the phagocytic and bactericidal capability of amoebocytes of anthozoans *in vitro*, using the beadlet anemone, *Actinia equina*, as a convenient experimental host. In particular, experiments were designed to measure the rate of uptake of bacteria by the cells, investigate the production of reactive oxygen species (ROS) by the phago-

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cytes, and ascertain the ability of the amoebocytes to inhibit bacterial growth *in vitro*. In many animals, O_2^- ions and other ROS generated by the cells during the respiratory burst are known to mediate bacterial killing by phagocytes (Fridovich, 1978; Nathan *et al.*, 1979). The process has been extensively studied in mammals, fish, and molluscs (Segal and Abo, 1993; Secombes *et al.*, 1988; Adema *et al.*, 1991; Pipe, 1992). It has also been reported for crustaceans (Bell and Smith, 1993) and ascidians (Bell and Smith, 1994) but not, as yet, for acoelomate animals.

Materials and Methods

Animals

Specimens of the beadlet anemone, *Actinia equina*, were collected from the lower shore of St Andrews Bay, Scotland, and maintained in a flow-through seawater aquarium (15°–20°C) for 1 week before use.

Extraction of amoebocytes

Amoebocytes were extracted from the mesenteric filaments, located at the base of the coelenteron, by making an incision into the center of the pedal disc and excising the filaments into a sterile 15-ml plastic centrifuge tube (Smith and Hutton, 1995). For the phagocytosis experiments, the filaments were mixed with about 3 ml of homologous fluid drained from the coelenteron and vigorously pipetted to dissociate the amoebocytes. Suspension of the cells in coelenteron fluid was found to be necessary to enhance the ability of the amoebocytes to spread on glass surfaces for the phagocytosis assays (see below). To avoid interference from endogenous antioxidant enzymes or other factors that might be present in coelenteron fluid, it was not used for the other experiments. Instead, the filaments were mixed with 3 ml of sterile 4% ocean salts (OS; Sigma, Poole, Dorset, UK), pH 7.0, and similarly pipetted. In each case, the amoebocytes were then allowed to stand for 5 min to sediment undissociated cells, the supernatant was removed, and the cell number was adjusted to a stock concentration of 5.2×10^4 ml^{-1} with OS. The amoebocytes were either used immediately or stored on ice for no longer than 30 min.

Bacteria

The gram-negative marine bacterium *Psychrobacter immobilis* (formerly *Moraxella* sp.; NCIMB 308) was used as a convenient test agent. It was chosen because it is easy to culture in the laboratory, is of a size readily distinguishable from intracellular inclusions in phagocytosis assays, and—although not pathogenic for anthozoans—is known to be susceptible to the antibacterial effects of marine invertebrate tissues or body fluids (Smith and Ratcliffe, 1978; Chisholm and Smith, 1992). It was grown to log phase in marine broth (MB; Difco, Detroit, Michigan) for 24 h at 20°C. Volumes of 15 ml were harvested, centrifuged at $400 \times g$, 4°C (10 min), and washed twice in sterile 3.2% NaCl. The bacteria were then resuspended in OS at a stock concentration of 5×10^8 ml^{-1}.

Measurement of phagocytosis in vitro

Amoebocyte suspension in homologous coelenteron fluid was pipetted, in 200-μl volumes, onto clean, pyrogen-free, glass coverslips in sterile tissue-culture-grade six-well plates (Sterilin, Stone, Staffs, UK). The coverslips were incubated at 20°C for 15 min to allow the amoebocytes to attach to the glass surface and spread. Each coverslip was then gently washed with two 1-ml volumes of sterile 0.22-μm-filtered OS and moistened with a fresh 50-μl volume of OS. The cells were challenged with 100 μl of washed bacterial suspension or, for the controls, 100 μl of sterile, filtered OS. The latter acted as an "uptake" control to permit direct visual comparison with the experimental cells. All monolayers were then incubated for 15, 30, or 45 min at 20°C. Uptake was not studied over longer incubation periods because preliminary evaluation of amoebocyte viability on the coverslips by eosin exclusion showed a decline from about 80% at 45 min to about 4% after 60 min (data not shown). The reason for the loss of cell viability on the monolayers at 60 min is unclear. The presence of the bacteria did not appear to be responsible because both control (saline-incubated) and experimental (bacteria-treated) preparations showed the same degree of amoebocyte survival (data not shown). Instead, the amoebocytes apparently spread so extensively on the glass surface that they lost structural integrity. After incubation, the monolayers were gently washed with three 1-ml changes of sterile, filtered OS and observed, unfixed, under phase contrast optics of a Leitz Diaplan microscope. Intracellular bacteria were distinguished from extracellular forms according to the criteria given in Smith and Ratcliffe (1978).

Superoxide anion assay and role of the respiratory burst in bacterial killing

The ability of the amoebocytes to produce O_2^- ions was investigated by using the ferricytochrome *c* reduction assay described by Pick and Mizel (1981). First, amoebocyte viability in OS in plastic microplate wells was checked by eosin exclusion at 0, 30, 60, 120, and 180 min at 20°C. These experiments confirmed that cell survival under these culture conditions remained above 85% for 3 h (data not shown). For the superoxide anion

assay, phorbol myristate acetate (PMA) (Sigma) or lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma) were used as elicitors, and the reactions were carried out in 96-well flat-bottomed microtiter plates, one per animal. For each treatment quadruplicate wells were prepared. All of the chemicals were made up in OS and were added to the wells in the order given: 50 μl of cytochrome *c* (from horse heart; Sigma) (final concentration in the well 64 μM); 25 μl of amoebocytes (final concentration 1.3×10^4 ml^{-1}); 25 μl of OS; and, immediately prior to reading, 25 μl of PMA or LPS. To determine the minimal concentration of the elicitors needed for clearly discernible cytochrome *c* reduction by the amoebocytes, the experiment was carried out using 1, 4, or 8 μg ml^{-1} of PMA or LPS, each in the presence of 0, 1, 4, or 8 μg ml^{-1} catalase (all final concentrations). Controls received 25 μl of OS in place of PMA or LPS. To ensure that no interaction occurred between the different compounds, all reagents were mixed independently with the ferricytochrome *c* at 20°C. The plates were read immediately at 550 nm on a microplate spectrophotometer (Dynatech, Billingshurst, West Sussex, England) against blanks composed of 25 μl amoebocytes and 100 μl OS. Additional readings were taken at 5-min intervals for 30 min. The experiment was repeated for a minimum of five animals and the mean absorbance determined for each treatment.

To establish that the reduction of cytochrome *c* by PMA-stimulated amoebocytes from *A. equina* (see Results below) was due to the production of ROS, additional experiments were run in which 25 μl of superoxide dismutase (SOD; an enzyme that converts O_2^- to H_2O_2), mannitol (a hydroxyl radical scavenger), or trifluoperazine (TFP; an inhibitor of NADPH oxidase)—all obtained from Sigma—was included in the reaction mixtures. The respective final concentrations of these chemicals were 1200 units ml^{-1}, 40 mM, and 10 μM. Preliminary experiments confirmed that amoebocyte viability was unaffected by these reagents at these doses. In each case, the PMA concentration in each well was 8 μg ml^{-1} (final), whereas that of LPS was 1 μg ml^{-1}. With LPS, 25 μl catalase (final concentration 1 μg ml^{-1}) was also included in the reaction mixture; whereas with PMA the catalase was replaced with OS. These concentrations were selected on the basis of the previous experiments to provide good reduction of ferricytocrome *c in vitro* (see above). The inhibitors were always added to the wells before the elicitor, and positive controls received 25 μl of OS in place of the inhibitor. For reagent controls, each chemical was incubated independently with non-stimulated amoebocytes and cytochrome *c*. The effect of the various inhibitors was read at 550 nm after 5 min at 20°C, and the change in absorbance from the resting 0 min level was calculated.

Measurement of bactericidal activity by viable amoebocytes in vitro

The ability of isolated amoebocytes from *A. equina* to inhibit the growth of *P. immobilis in vitro* was examined using a modification of the microplate assay described by Peck (1985) for macrophages. Fifty microliters of amoebocyte suspension in OS (stock concentration 5×10^4 ml^{-1}) was added to wells of 96-well flat-bottomed microtiter plates. Experimental wells were then given 50 μl of prepared bacterial suspension in OS (diluted to a stock of 2×10^5 ml^{-1}) and 25 μl of MB made to manufacturer's specifications (Difco, Detroit, Michigan); amoebocyte controls received 50 μl of sterile, filtered OS and 25 μl of MB. An additional series of wells (the bacteria controls) contained 50 μl of bacterial suspension, 50 μl of OS, and 25 μl of MB. The plates were incubated for 3 h at 20°C and the amoebocytes were then lysed by addition of 100 μl of a stock solution of 0.2% Tween (Sigma) to each well. This step was included to prevent further generation of ROS by the experimental cells. Next, 50 μl of bacterial suspension (stock solution 2×10^5 ml^{-1}) was added to each amoebocyte control well, with 50 μl of sterile, filtered OS added to each of the experimental wells and each of the bacteria controls. Finally, 150 μl of MB was given to every well to support the growth of surviving bacteria. The absorbance of the wells was read at 570 nm on a microplate spectrophotometer (Dynatech) (time T_0). The plate was incubated for a further 18 h at 20°C and read again at 570 nm (time T_{18}).

Bacterial growth was seen as an increase in absorbance over 18 h at 20°C. The effect of the amoebocytes on bacterial growth was calculated as the survival index (SI). This was derived as follows:

$$\text{SI} = \frac{\text{absorbance at 570 nm at time } T_{18}}{\text{absorbance at 570 nm at time } T_0} \times 100$$

With this notation, SI values greater than 100 indicate growth, whereas those below 100 indicate an antibacterial effect (*i.e.*, growth inhibition or bacterial killing).

Antibacterial activity of amoebocyte lysate supernatant (ALS)

To clarify whether the antibacterial properties of *A. equina* amoebocytes are due to soluble factors or to membrane-bound proteins, the effect of lysate supernatants of the amoebocytes (ALS) on the test bacterium, *P. immobilis*, was examined *in vitro*. Each ALS sample was prepared by homogenizing washed amoebocytes in 3 ml of OS in a glass piston tissue grinder (15 min, 4°C) followed by centrifugation at 42,000 $\times$ *g* (20 min, 4°C) to sediment cell debris. The resulting supernatant (=ALS)

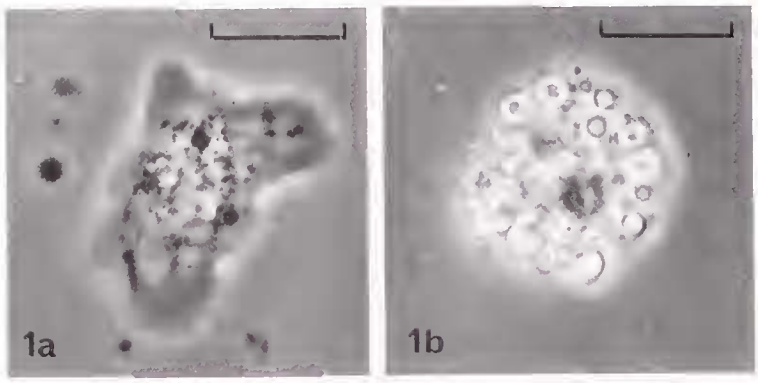

Figure 1. Amoebocytes of *Actinia equina* isolated from the mesenteric filaments after 45-min incubation on glass coverslips. (a) A spread hyaline (phagocytic) type cell containing numerous small, usually nonrefractile, inclusions. Scale bar = 5 μm. (b) An intact granular cell containing many large, highly refractile granules. Scale bar = 5 μm.

was stored on ice until use. Protein in the ALS was determined by the method of Bradford (1976) with bovine serum albumin as standard and routinely adjusted by dilution with OS to 2 mg ml^{-1} before use. Aliquots (80 μl) of the ALS were then dispensed in quadruplicate wells of a 96-well (flat-bottomed) microtiter plate and mixed with 10 μl of MB and 10 μl of *P. immobilis* (stock concentration 2×10^5 ml^{-1}). For controls, 80 μl of OS was substituted for the ALS. The absorbance of each of the control and experimental wells was read at 570 nm after incubation at 20°C for 0, 3, 6, 12, and 24 h.

Because the bactericidal assays with whole amoebocytes indicated that the response involves lysis (see below), we undertook to ascertain whether the antibacterial properties of *A. equina* ALS are due at least in part to lysozyme activity. A modification of the procedure described in Findlay and Smith (1995) was used. Briefly, ALS samples were prepared as above but in salt-supplemented phosphate-buffered saline (ssPBS; 20 m*M* $Na_2HPO_4 \cdot 2H_2O$; 45 m*M* KH_2PO_4; 0.1 *M* NaCl; pH 6.5). They were serially diluted to protein concentrations of 1500, 1000, 500, or 200 μg ml^{-1}, and 100 μl of each dilution was mixed with 900 μl of *Micrococcus luteus* cell walls (Sigma) previously suspended in ssPBS to give an absorbance of 0.5 at 570 nm. An extra tube containing PBS in place of ALS was set up as the negative control. To provide both positive controls and a standard curve by which any lysozyme-like activity in *A. equina* ALS could be quantified, a series of hen egg white lysozyme (Sigma) solutions in ssPBS at the same protein concentrations as the ALS were set up in parallel. All tubes were incubated at 25°C for 30 min with shaking and then read at 600 nm. Lysis in each experimental tube was calculated as the percentage of its positive control. Again the experiment was repeated three times with fresh ALS samples.

Statistical analysis

Differences between each experimental treatment and its control were analyzed by paired Student's *t* test. Differences were considered significant when $P \leq 0.01$ (Sokal and Rolhf, 1981).

Results

Phagocytosis in vitro

Observation of the amoebocyte suspension prepared from the mesenteric filaments of *A. equina* under phase contrast optics revealed the presence of two morphologically distinct cell types. One, the hyaline cells, was seen to contain numerous small, nonrefractile granules (Fig. 1a); the other, the granular cells, contained many large, highly refractile granules (Fig. 1b). The proportion of hyaline to granular cells on the monolayers tended to differ from animal to animal but was usually about 5 : 1 (data not shown). Both types were seen to attach to glass coverslips, but only the hyaline cells exhibited marked spreading, and this was largely dependent upon the presence of coelenteron fluid. The granular cells showed a limited ability to spread on glass, even in the presence of coelenteron fluid. The hyaline cells were also the only amoebocyte type seen to ingest bacteria *in vitro*. After 15 min the percentage of these amoebocytes containing one or more bacteria was 20% ± 1.8% (Fig. 2). By 45 min this had increased significantly to a maximum of 39.6% ± 1.4% ($P = 0.002$). Intracellular bacteria were not seen in any of the cells on the control, OS-incubated, monolayers.

Superoxide anion assay

Figure 3 shows the effect of different concentrations of PMA or LPS on the *in vitro* reduction of ferricytochrome

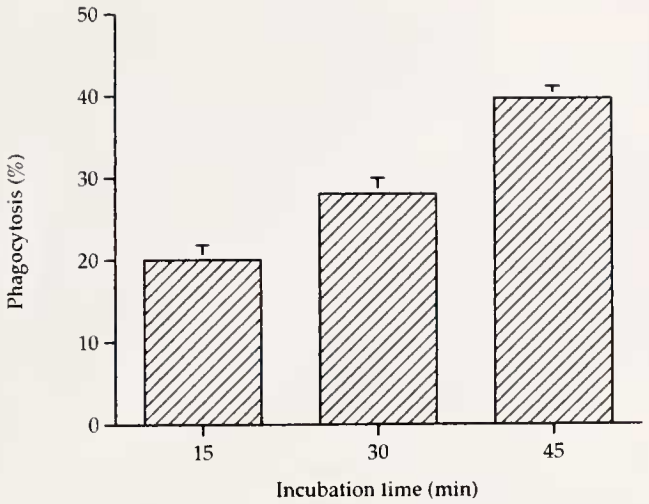

Figure 2. Percentage phagocytosis of *Psychrobacter immobilis* by isolated amoebocytes from *Actinia equina in vitro*. Cell monolayers, prepared on glass coverslips, were challenged with 100 μl of washed *P. immobilis* (stock concentration 5×10^7 in ocean salts [OS]). Controls received 100 μl of sterile, filtered OS and all cultures were incubated for 15, 30, or 45 min at 20°C. The percentage of cells containing one or more intracellular bacteria was determined by observation of washed, unfixed monolayers under phase contrast optics. Values given are means ± SE; $n = 5$.

c by the amoebocytes of *A. equina* in the presence of varying catalase concentrations after 5 min. Of all the elicitor-catalase combinations tested, absorbances were highest with PMA at 8 μg ml^{-1} without catalase (Fig. 3a), or LPS at 1 μg ml^{-1} with catalase at a final concentration of 1 μg ml^{-1} (Fig. 3b). This promotional effect of catalase was seen only at a concentration 1 μg ml^{-1} and was most marked with 1 μg ml^{-1} LPS (Fig. 3b). The reason that catalase at 4 or 8 μg ml^{-1} produced an LPS-induced ferricytochrome *c* reduction similar to, or slightly less than, that of the catalase-free condition is unknown. Catalase had only a marginal effect on PMA-elicited cytocrome *c* reduction (Fig. 3a) and therefore was omitted from subsequent experiments with PMA. Neither PMA nor LPS exerted a change in absorbance in control wells lacking *A. equina* amoebocytes (data not shown).

Figure 4 shows the change in absorbance at 550 nm over 30 min for amoebocytes incubated with PMA (8 μg ml^{-1} final concentration) or LPS (1 μg ml^{-1} final concentration) with or without 1 μg ml^{-1} catalase, compared to the controls, in which PMA or LPS and catalase were replaced with OS. The absorbance of the wells at 5 min after treatment with PMA (with or without catalase) or LPS (1 μg ml^{-1} plus catalase) was significantly higher than that of their respective OS-incubated controls ($P < 0.0001$ for PMA without catalase, $P < 0.0001$ for PMA with 1 μg ml^{-1} catalase, and $P < 0.0001$ for LPS with 1 μg ml^{-1} catalase; all compared to the OS-treated samples at 5 min) (Fig. 4a, b). The slight increase in absorbance obtained at 5 min with LPS (1 μg ml^{-1} without catalase) was not significant at the 5% level compared to the OS controls ($P = 0.643$) (Fig. 4b). Over the next 25 min, the absorbance of all wells declined, and with PMA or LPS, both minus catalase, the absorbances fell to values close to those of the OS-treated controls (Fig. 4a, b). In contrast, PMA or LPS, both plus catalase, produced a much less dramatic decline in absorbance, and the 30-min absorbance values remained significantly above the OS controls ($P < 0.0001$ for PMA; $P < 0.0001$ for LPS) yet below their respective values at 5 min ($P < 0.0001$ for PMA; $P < 0.0001$ for LPS) (Fig. 4a, b).

Thus, exposure of *A. equina* amoebocytes to PMA or LPS seems to result in the reduction of ferricytochrome *c*, indicating that the cells were stimulated to produce ROS through a respiratory burst. The reason for the decline in the absorbance in the control and PMA or LPS (both minus catalase) wells is unknown. The phenomenon may have been due to to re-oxidation of ferricytochrome *c*, perhaps by hydrogen peroxide produced by the cells. Certainly, less re-oxidation of cytochrome *c* occured where catalase, an enzyme that converts hydrogen peroxide to water and molecular oxygen (Vandewalle and Petersen, 1987), was present. In the case of the controls, in which catalase and elicitor were absent, the change in absorbance must have been due to spontaneous oxidation of the substrate over the 30-min incubation period.

In the next series of experiments, inclusion of exogenous SOD in the reaction mixtures was found to significantly reduce the stimulatory effect of PMA on ferricytochrome *c* reduction ($P < 0.038$ for the PMA + SOD-treated cells compared to the cells treated with PMA + OS) (Fig. 5). Because SOD brings about the conversion of O_2^- ions to H_2O_2, this observation offers evidence that the reduction in ferricytochrome *c* by *A. equina* amoebocytes induced by PMA is due to the generation of superoxide ions *in vitro*. Likewise, mannitol and TFP significantly abrogated the effect of PMA on the absorbance of the experimental wells ($P < 0.0001$ for each reagent compared to the PMA-treated amoebocytes incubated with OS) (Fig. 5). The effect of these chemicals on PMA-induced reduction of ferricytochrome *c* by the amoebocytes further indicates that the phenomenon involves NADPH oxidase, OH^- ions, and H_2O_2.

Bactericidal activity by viable amoebocytes in vitro

Washed *P. immobilis* incubated with viable amoebocytes showed significantly poorer growth then bacteria incubated with OS and MB only ($P < 0.0001$) (Fig. 6). However, because a reduced SI was also derived for the control (Tween 20 lysed) amoebocytes (Fig. 6), it is impossible to conclude that bacterial killing was due to metabolic products generated by viable amoebocytes. In-

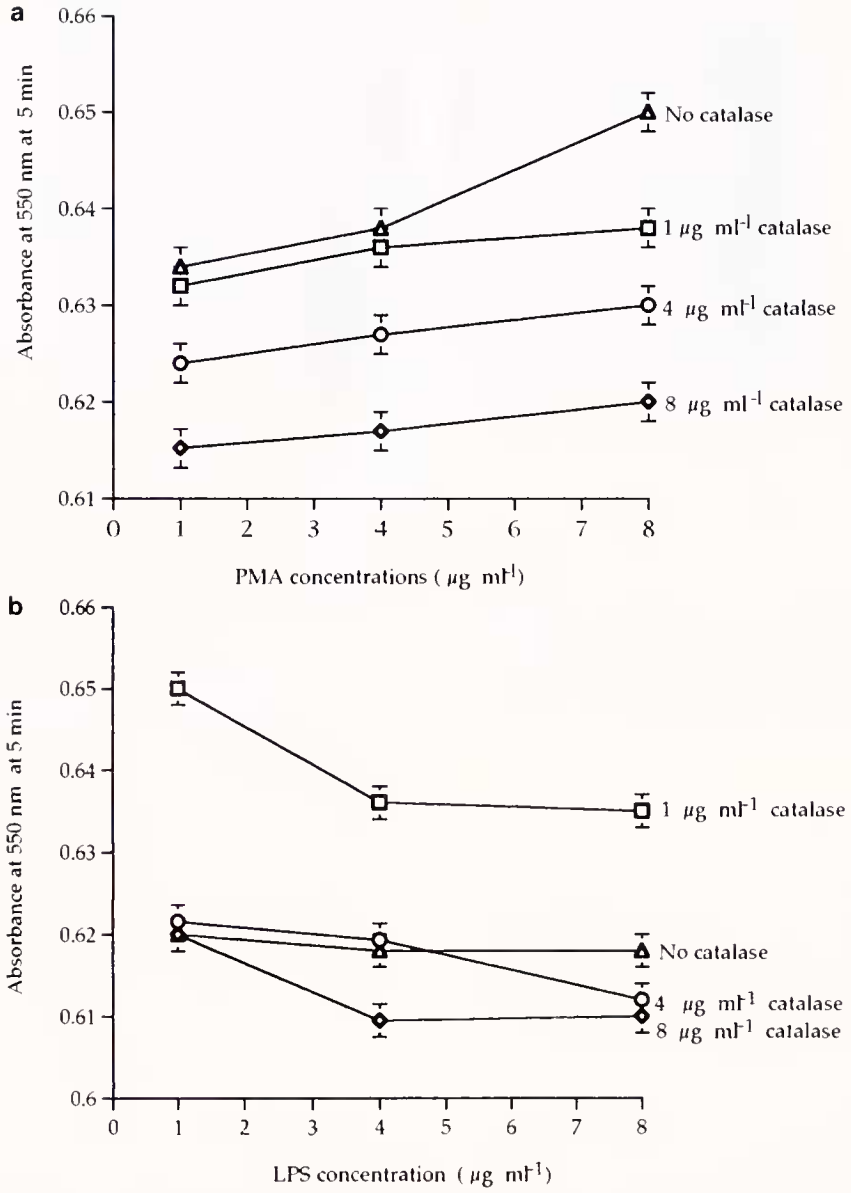

Figure 3. Effect of different phorbol myristate acetate (PMA), lipopolysaccharide (LPS), and catalase concentrations on the reduction of ferricytochrome *c* by isolated amoebocytes from *Actinia equina in vitro*. Twenty-five microliters of washed amoebocytes in ocean salts (OS; final concentration 1.3×10^4 ml^{-1}) was incubated with 25 μl of PMA (a) or LPS (b) (both at final concentrations of 1, 4, or 8 μg ml^{-1}), 25 μl of catalase (final concentrations 0, 1, 4, or 8 μg ml^{-1}), 25 μl of OS and 50 μl of ferricytochrome *c* (final concentration 64 μ*M*). For controls, the PMA or LPS and catalase was replaced by 50 μl of OS and the absorbance of each well was measured at 550 nm after 5 min. Values given are means ± SE; $n = 5$.

stead, the cells appear to release one or more bactericidal factors upon exposure to Tween 20. Given that the SI for both experimental and control amoebocytes was below 100 ($P < 0.0001$) (Fig. 6), at least one of these factors is a lysin.

Antibacterial activity of ALS

That the antibacterial activity by *A. equina* amoebocytes was due to one or more soluble, rather than membrane-bound, factors was confirmed by the results of the experiments with ALS. Experimental (ALS-incubated) bacteria showed significantly poorer growth than control *P. immobilis* incubated in OS supplemented with MB ($P < 0.0001$ after 18-h incubation at 20°C) (Fig. 7). Unfortunately, this assay failed to confirm that the antibacterial effect of *A. equina* ALS involves lysis, possibly because the rapid growth of surviving bacteria would have masked any reduction in bacterial turbidity

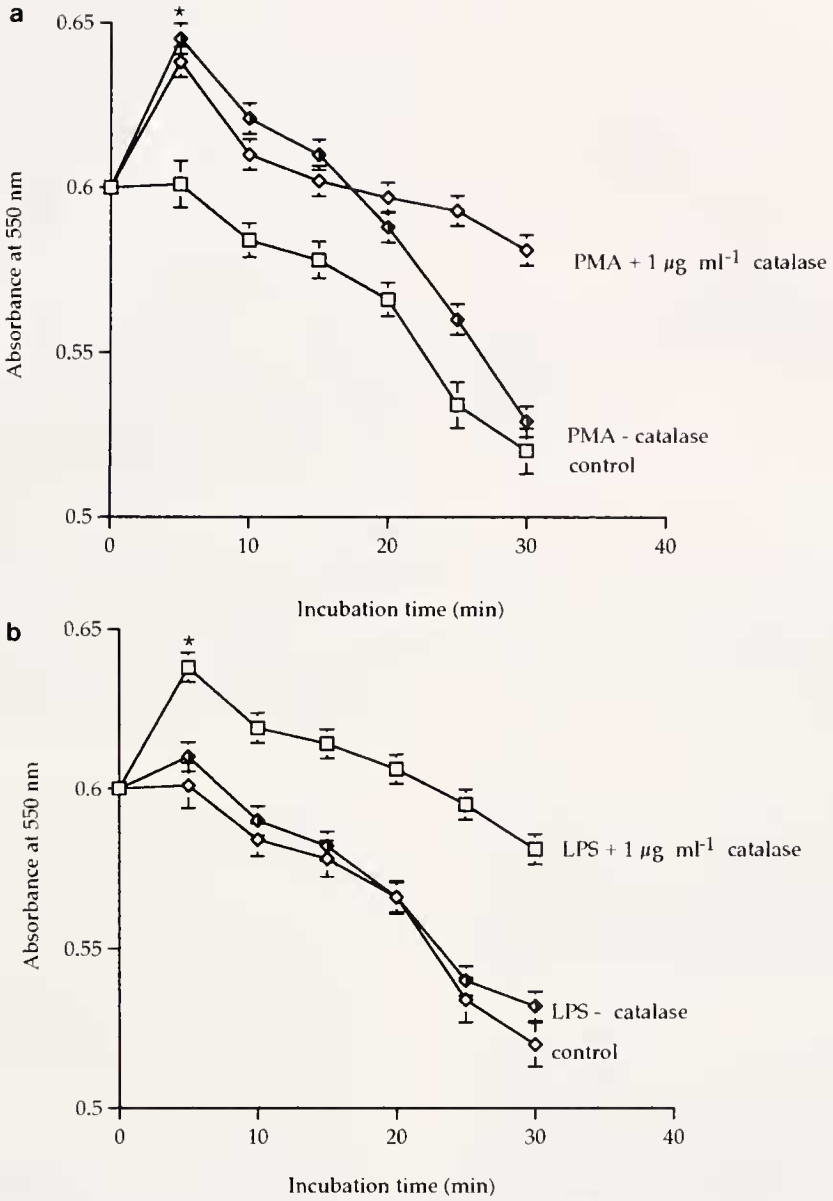

Figure 4. Reduction of ferricytochrome *c* by amoebocytes from *Actinia equina in vitro*. Twenty-five microliters of washed cells in ocean salts (OS; final concentration 1.3×10^4 ml^{-1}) was incubated with 25 μl of phorbol myristate acetate (PMA; final concentration 8 μg ml^{-1}) (a) or lipopolysaccharide (LPS; final concentration 1 μg ml^{-1}) (b), 25 μl of OS and 50 μl of ferricytochrome *c* (final concentration 64 μ*M*). For controls, PMA or LPS was replaced with 25 μl of OS. An extra series of wells, in which 25 μl of catalase (final concentration 1 μg ml^{-1}) was substituted for the OS, was also included for both PMA- and LPS-treated cells to enable the effect of catalase on ferricytochrome *c* reduction to be monitored over 30 min. The absorbance of each well was measured at 550 nm at 5-min intervals for 30 min. Values given are means ± SE; $n = 5$. An asterisk (*) indicates that differences between experimental and control values are significant at the 1% level.

compared to the time zero controls. Our subsequent experiments to test for lysozyme-like activity also failed to reveal significant lysis of *Micrococcus luteus* cell walls at any of the ALS concentrations tested, indicating that lysozyme is not one of the main agents involved in bacterial killing.

Discussion

The results of this study show that amoebocytes isolated from the mesenteric filaments of *A. equina* are actively phagocytic *in vitro* and produce substances that are able to inhibit the growth of gram negative bacteria

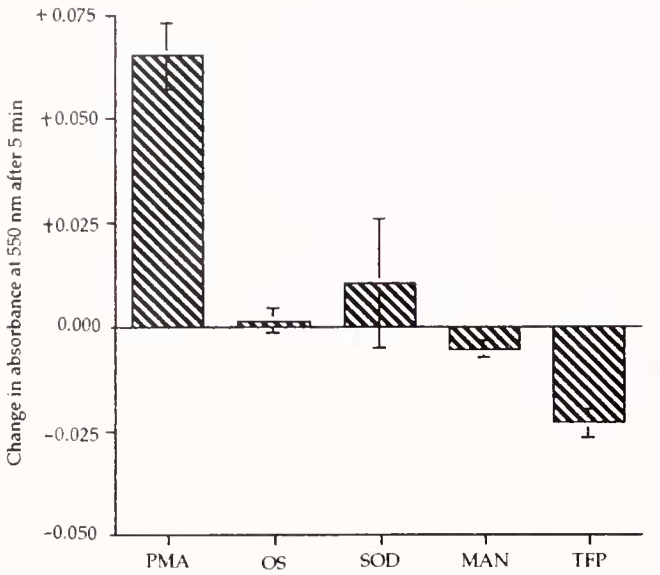

Figure 5. Change in ferricytochrome *c* reduction by phorbol myristate acetate (PMA) stimulated amoebocytes from *Actinia equina* by treatment with superoxide dismutase (SOD), mannitol (MAN), or trifluoperazine (TFP) *in vitro*. Twenty-five microliters of amoebocyte suspension in ocean salts (OS; final concentration 1.3×10^4 ml^{-1}) was challenged with 25 μl of PMA (final concentration 8 μg ml^{-1}), 50 μl of ferricytochrome *c* (final concentration 64 μ*M*), and 25 μl of OS (controls) or 25 μl of the appropriate reagent (experimentals), at final concentrations of 1200 units ml^{-1}, 40 m*M*, or 10 μ*M*, respectively. All samples were incubated for 5 min and read at 550 nm. The absorbance of each well was measured at 550 nm after 5 min at 20°C and the change in absorbance from the resting value at 0 min calculated. Values given are means ± SE; $n = 5$. Differences between the experimental (*i.e.*, SOD-, MAN-, or TFP-treated) and control (*i.e.*, PMA-stimulated) amoebocytes was significant at the 1% level.

within 3 h. There have been few previous analyses of the bactericidal properties of anthozoan tissues and none aimed at examining the mechanism for antibacterial effect. An early study by Burkholder and Burkholder (1958) screened for antimicrobial activity in gorgonians and scleractinian corals; although activity was detected in several species, the authors were unable to identify the tissue responsible or to establish whether activity was mediated by the symbiotic zooxanthellae or the host tissue. Astley and Ratcliffe (1989) examined the antimicrobial and agglutinating properties of mucus from *Metridium senile* but did not consider activity in the tissues. In contrast, a more recent investigation by Kim (1994) was directed towards the antibacterial responses of gorgonian tissues, and although activity was found against non-marine bacteria, the assays were made on whole-body extracts only. To the best of our knowledge, this is the first detailed report confirming the antibacterial capability of anthozoan amoebocytes *in vitro*, and the first to show that large numbers of phagocytically competent amoebocytes can be isolated from the mesenteric filaments.

The absence of a coelom and distinct blood vascular system in anthozoans has tended to exclude these animals from the realms of comparative hematology. In place of circulating blood cells, the wandering amoebocytes of the mesoglea have been regarded as primordial immunocytes responsible for non-self surveillance and host defense (Phillips, 1963; Bigger and Hildemann, 1982), an idea based mainly on observations that these cells accumulate at wound sites or foreign tissue grafts (Young, 1974; Patterson and Landolt, 1979; Bigger and Hildemann, 1982; Bigger and Olano, 1994). Research into the true biological roles and phylogenetic status of the mesogleal amoebocytes is hampered by the small, and often variable, number of the cells in anthozoans and the difficulty of their isolation for *in vitro* analysis (Patterson and Landolt, 1974; Van der Vyver, 1981). The present study avoided these problems by using amoebocyte populations extracted from the mesenteric filaments. The exact relationship of the mesenteric filament amoebocytes to those in the mesoglea is unclear. Authors disagree about the origin and primary role of the amoebocytes in the mesoglea (Chapman, 1974; Young,

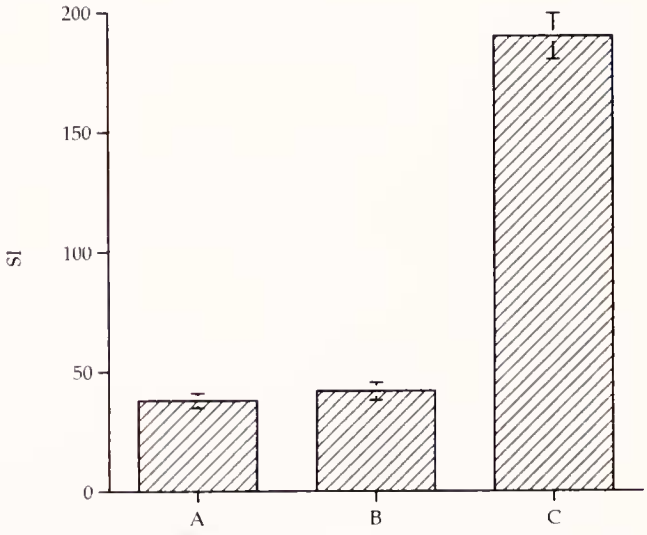

Figure 6. Antibacterial activity of intact, viable amoebocytes from the mesenteric filaments of *Actinia equina in vitro*. The experimental wells (A) and the control wells (B) each contained 50 μl of amoebocytes and 25 μl of marine broth (MB). Fifty microliters of *Psychrobacter immobilis* was added to A, whereas 50 μl of ocean salts (OS) was added to B. The bacteria controls (C) comprised 50 μl of *P. immobilis*, 50 μl of OS, and 25 μl of MB. After 3 h each well received 100 μl of Tween 20 (100 μl); 50 μl of OS was then added to A and C, while 50 μl of *P. immobilis* were added to B. Finally, 150 μl of MB was pipetted into every well to support the growth of surviving bacteria, and the samples were incubated for a further 18 h before measurement of turbidity at 570 nm. The survival index (SI) was calculated as described in the Materials and Methods. Values given are means ± SE; $n = 5$. Differences between A and C are significant at the 1% level, whereas differences between A and B are not significant at the 5% level.

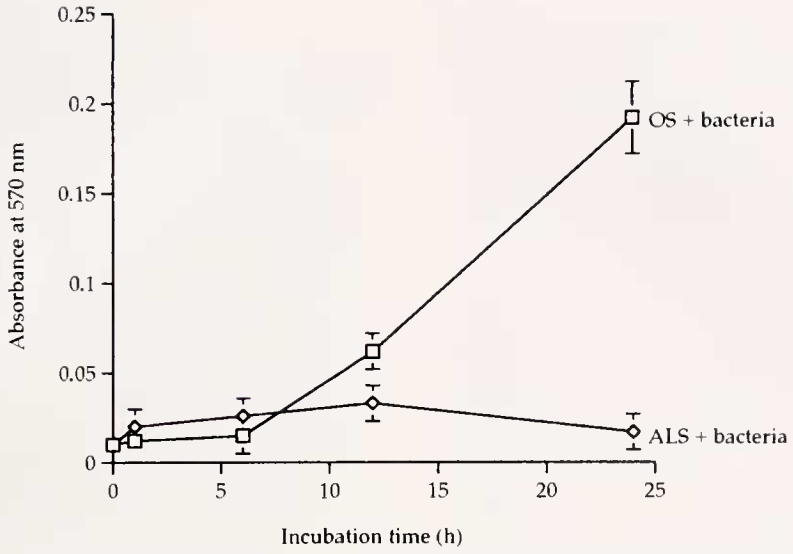

Figure 7. Antibacterial activity of amoebocyte lysate supernatant (ALS) from *Actinia equina* against *Psychrobacter immobilis in vivo*. Ten microliters of *P. immobilis* (stock concentration 2×10^5 ml^{-1}) was incubated with 80 μl of ALS (stock protein concentration 2 mg ml^{-1}) and 10 μl of marine broth (MB) at 20°C. For controls, 10 μl of the bacteria was incubated with 80 μl of OS and 10 μl of MB. After 12 h and 24 h, the absorbances were read at 570 nm. Values given are means ± SE, $n = 5$. Differences between the control and experimental values are significant at the 1% level.

1974; Willmer, 1991), although these cells are thought to participate in nutrient storage, transport, and digestion (Chapman and Pardy, 1972; Van-Praët, 1985; Shick, 1991). Crucially, Young (1974) has proposed that they are important in wound repair and migrate to sites of injury from a reservoir in the endodermal mesenteries. Our light microscopical observations of the mesenteric filament cells of *A. equina* agree with descriptions of amoebocytes in the mesoglea of other anthozoan species (Westfall, 1966; Singer, 1971; Chapman, 1974; Bigger and Hildemann, 1982). We show, unequivocally, that the hyaline amoebocytes, at least, are capable of phagocytosis, thus confirming their potential to sequester and inactivate foreign material from the endoderm. The finding that the mesenteric filament amoebocytes in *A. equina* also exhibit antibacterial activity signifies that they must play a role in host defense, and thus constitute a valuable source of immunocompetent effector cells for *in vitro* analyses.

Interestingly, the presence of two morphological categories of amoebocytes (hyaline and granular cells) noted for *A. equina* in the present investigation is comparable to the situation in most other invertebrate phyla, in which phagocytes and nongranular-type cells occur almost universally (Ratcliffe and Rowley, 1979). In addition, the amoebocytes of *A. equina* appear able to attach to glass surfaces, to exhibit (a variable degree of) cell spreading, and to ingest foreign particles *in vitro;* these features are also shown by certain blood cell types in many invertebrate species. However, unlike the cells in other groups, in *A. equina* amoebocyte spreading seems to be influenced by the coelenteron fluid. As yet, we do not know how this fluid affects spreading behavior, but unpublished observations in our laboratory indicate that it involves one or more heat-labile, dialyzable, and calcium-dependent "factors." Surprisingly, spreading of the amoebocytes, especially the hyaline cells, on glass surfaces did not appear to be self-limiting as it is with the blood cells of other invertebrates: a sizable proportion of these cells were seen to disintegrate after 45–60 min on the monolayers. To avoid this problem in the superoxide anion and bactericidal assays, where more prolonged incubation times were required, cell spreading—and hence cell distintegration—was minimized by incubating the amoebocytes in OS and in wells of tissue-culture-grade plastic microtiter plates instead of on glass coverslips.

Notwithstanding, the phagocytic vigor of *A. equina* amoebocytes *in vitro* is within the range of values obtained for the phagocytes from coelomate invertebrates cultured under similar conditions. For example, the percentage phagocytosis of *P. immobilis* by amoebocytes from the solitary ascidian *Ciona intestinalis* is about 40% (Smith and Peddie, 1992), and that of phagocytes from the starfish *Asterias rubens* is about 42% (Smith and Saunders, unpub. obs.). The hemocytes from decapod crustaceans tend to show lower rates, at about 15%–20% (Smith and Ratcliffe, 1978; Smith and Söderhäll, 1983), and those of annelids display rates as low as 3%–5% (Fitz-

gerald and Ratcliffe, 1982). Another feature that the amoebocytes of *A. equina* appear to share with the blood cells of other animals is the ability to reduce ferricytochrome *c* following stimulation with PMA or LPS. Moreover, as the response to PMA was found to be impaired by inclusion of SOD, mannitol, or TFP, the reduction of ferricytochrome *c* must be due to the production of superoxide anions and other ROS through an NADPH oxidase-mediated respiratory burst. As far as we are aware, the respiratory burst has not been described previously for a member of the Cnidaria. Importantly, the speed of the response in *A. equina* is comparable with that in other animals; ROS production has been reported to occur within 10 min in oyster phagocytes (Takahashi *et al.*, 1993), 15 min in fish macrophages (Secombes *et al.*, 1988), and 2 min in human neutrophils (Ryan *et al.*, 1990).

For many animals, ROS generated through the respiratory burst have been shown to effect intra- or extracellular microbial killing by the phagocytes (Nathan *et al.*, 1979; Sharp and Secombes, 1993; Segal and Abo, 1993). Whether this is true for *A. equina* could not be ascertained in the present study because the experiments to measure the bactericidal properties of viable amoebocytes were confounded by the release of soluble factors from both the control and experimental cells upon treatment with Tween 20. As yet the nature and mechanism of action of the agent or agents responsible remain unknown. The large decrease in the SIs of the bacteria incubated with Tween 20-treated amoebocytes indicates that the response could involve a lysin. One of the best-known lytic agents in coelomate body fluids is lysozyme. This appears to be present in a wide range of phyla and is widely believed to aid in the nonspecific antibacterial defenses of both vertebrates and invertebrates (Millar and Ratcliffe, 1994). There have been comparatively few reports of lysozyme in acoelomates, although Phillips (1963) has described a series of experiments which indicate that lysozyme-like enzymes occurs in the mucus of *A. elegantissima*. Our assays with *A. equina* ALS did not, however, reveal lysozyme-like activity, as determined by lysis of *M. luteus* cell walls in *A. equina* amoebocytes. Thus the presence or absence of lytic agents in anthozoan amoebocytes remains equivocal. We are undertaking detailed characterization of the antibacterial activity in *A. equina* to establish the biochemical identity, cellular location, and biological effect of the factor or factors involved.

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